The Effect of Collagen II and TGF-b Growth Factor Presence in 3D Alginate Bead Culture on the Differentiation of Mesenchymal Stem Cells to Chondrocytes to Generate Repair Tissue

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Introduction

The regeneration of native tissue had arisen as a new approach to the treatment of severe trauma. Scientists have been tweaking the properties of scaffolds (the initial support structure), cytokines and cell type to create an injectable or implantable method of tissue regeneration. Specifically, modification of scaffold elasticity, strength, degradation, motifs and release factors has been explored to promote chondrocytes growth.

Previous research has demonstrated that a combination of both Collagen II and TGF-b growth factor on the scaffold stimulated the most chondrocyte presence. Based on this information it followed that observation of how little Collagen II and TGF-b growth factor is needed to stimulate MES differentiation was necessary. The initial Collagen II and TGF-b levels were taken from the Bosnakovski el al. paper, from this a 1:5 dilution was tested. Should the lower concentration stimulate differentiation, material use and consequentially cost would be reduced; two important factors should this method go to clinical trials. It would be also interesting to see if Collagen II and TGF-b would stimulate differentiation of MES cells, not just continued growth of chondrocytes as stem cells have a much higher proliferation rate, in turn repairing the damaged tissue faster.

Results

Cell Culture Conditions

Following the methods used in the Bosnakovski el al. paper, Collagen II and TGF-b concentrations were added to 1% alginate made by Sigma Aldrich and a differentiated medium. Two samples and two no cell controls (just alginate and alginate with Collagen II) were tested; in the first a 0.3% solution of Collagen II and 10ng/ml of TGF-b were added, the second sample was a 1:5 dilution of the additives. Cells were plated at a concentration of 5*10e6 cells/ml onto the prepared medium.

LIVE/DEAD Assay from Molecular Probes

As a check for cell viability and growth a Live/Dead Assay was performed. Cells were dyed with both SYTO 10 and ethidium homodimer-2 and fluorescent readings were taken. Two methods of analysis, manual and automated were utilized to obtain a cell count.

The SYTO 10 dye fluoresced all cells while ethidium homodimer-2 fluoresced only dead cells. Based on this fact, the number of cells in each dye was manually counted, those dyed with ethidium homodimer-2 were subtracted from the number of SYTO 10 dyed cells. The fluorescence data was also analyzed through ImageJ. The cell counts for both methods were quite similar; the ImageJ count was 73 cells and the manual count was 86 cells.

RT-PCR for Analysis of Collagen II:I Ratios and Evaluation of Cell Type

After 11 days of growth the cells were lysed and their RNA was extracted for analysis through RT-PCR, 100ng of RNA was used. The Collagen II and I levels were observed along with a housekeeping cDNA for GAPDH (see Figure1). Primers for the RT-PCR were taken from the Ikenooue et al. paper; however these primers were made for adult human cartilage and not bovine, as needed. A BLAST of these primers against the bovine genome was performed to obtain bovine primers.
The cDNA bands from the 1:1 and 1:5 samples were compared through ImageJ to those obtained from chondrocytes and MES cells to determine cell type. The Collagen II/I ratios were calculated through Excel by subtracting the background intensity of the gel from the band intensities and subsequently dividing those intensities. The 1:1 sample showed a Collagen Ratio slightly less then that expressed by chondrocytes; the 1:5 intensity was similar to MES cells demonstrating little differentiation in that sample (see Table 2).

Table 2: Collagen II: I Ratios from RT-PCR

Sample	Collagen II/ Collagen I
Chondrocyte	0.3239
MES	2.304e-9
One to One	0.1773
One to Five	1.8122e-5

ELISE Assay for Analysis of Collagen II:I Ratios and Evaluation of Cell Type

Alongside the RT-PCR an Indirect ELISA Assay was used to determine Collagen Ratios. Four samples, 1:1, 1:5, collagen II and alginate, and alginate only, were run in duplicate along side a standard curve (see Figure 2). The fluorescence readouts were averaged and background was subtracted out. The collagen concentrations were then determined according to a best fit of the collagen gradients. The one to one concentration is much higher than the one to five, which is similar to the RT-PCR results. The one to five sample showed lower collagen II levels than both without cell controls.

Table 3: Collagen II:I Ratios from ELISA Assay

Sample	Collagen II/ Collagen I
One to One	1.300
One to Five	0.5207
Collagen II + Alginate	0.746
Alginate Only	0.626

Discussion

According to the Bosnakovski el al. paper a 0.03% Collagen II solution and 10ng/ml of TGF-b added to 1% alginate, lead to increased chondrocyte number. Our research observed whether these additives would also stimulate MES differentiation to chondrocytes. As the ELISA and RT-PCR assay showed Collagen II levels were close to those in the pure chondrocyte sample, demonstrating differentiation. The ability to differentiate MES cells to chondrocytes is extremely important as although there are initially fewer MES cells they grow exponentially, unlike initially transplanted chondrocytes, which exhibit minimal growth.

A second aspect of our research was to see whether a decreased combination of Collagen II and TGF-b factor could stimulate differentiation, as less use of materials would be optimal. A 1:5 dilution of the initial Collagen II and TGF-b was administered to 1% alginate. However, the Collagen ratios from this sample showed little differentiation, the ratios were similar to undifferentiated MES cells.

Future Research

For future research I would like to see if a higher dose of Collagen II and TGF-b would lead to increased differentiation. Consequentially, if I had a lot of time and resources I should like to run a concentration gradient of these additives to see how much differentiation can be increased and how little of these factors can stimulate chondrocyte growth. As this experiment only allowed the cells to grow for 11 days, I should like to test a longer time course, perhaps with longer time to grow the 1:5 sample may have expressed chondrocytes. Alternatively, by viewing the cells over a longer time course, one could observe any dedifferentiation.

It would be interesting to try more quantitative method of Collagen II:I Ratio analysis. I should like to run the samples on qPCR as this method includes efficiency correction and one could observe the real time results.

References

1.)Ikenoue T, Trindade MC, Lee MS, Lin EY, Schurman DJ, Goodman SB, Smith RL. *Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro*. J Orthop Res. 2003 Jan;21(1):110-6.

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